Chapter

Gene and Cell Therapy in Dental Tissue Regeneration

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Abstract

Advanced therapies hold substantial promise for the treatment of periodontal conditions. Gene therapy has the potential to transfer "therapeutic" genes, which express proteins such as bone morphogenetic proteins, osteoprotegerin, and tissue nonspecific alkaline phosphatase, which is deficient in patients with hypophosphatasia, a condition that affects mineralization of teeth and bone. Transferred genes may also express platelet-derived growth factor, which modulates the growth of periodontal tissue and the alveolar bone. As regards cell therapy, several clinical trials have shown that mesenchymal stem cells, when used with different kinds of scaffolds to enable the required three-dimensional environment, possess a bone regeneration potential that is particularly useful in such disorders as osteoporosis and osteonecrosis, or for regenerating alveolar bone (osseointegration) prior to placing a dental implant. However, much work is still required before these new therapies become true alternatives in routine clinical dental practice. Medical advances require investments, which are usually influenced by the priorities of both politicians and society at large. This will contribute to promoting innovation, efficient treatments, medium- and long-term savings, and a higher quality of life.

Keywords: Advanced therapies, gene therapy, cell therapy, tissue regeneration, alveolar bone, mesenchymal stem cells, biomaterials and scaffolds, implants, Good Manufacturing Practices, clinical dental practice

1. Introduction

Advanced therapies encompass a group of novel and innovative pharmacological procedures including gene therapy, cell therapy and regenerative medicine. Their goal is to provide curative treatment for diseases or dysfunctions that can currently be managed only with palliative care. According to the European Medicines Agency (EMA), advanced therapies are medicines for human use that are based on genes, tissues, or cells, offering ground-breaking new opportunities for the treatment of various diseases of different aetiologies, ranging from hereditary to acquired, such as pathogen-induced infections or cancer [1].

Gene-based medications and procedures are based on "therapeutic" genes whose effect may be curative, but also prophylactic or even diagnostic. By using different transfection methods, advanced therapies aim to insert "recombinant" genes into a diseased cell or organism in order to replace or repair defective genes [2, 3].

Cell therapy procedures involve any cells that no not have the potential to contribute to the genetic material of the subject's offspring (germ-line cells). After minimal manipulation, the cells are "implanted" "or transplanted" autologously (same individual), allogeneically (different individual of the same species) or xenogeneically (individual of a different species) into an organism in order to restore a diseased structure or an impaired function [4, 5].

Regenerative medicine or tissue engineering, for its part, is based on restoring function through the transplantation of cells, tissues, or organoids [6, 7]. Although regenerative medicine procedures are at the present time still at the early stages of development, very promising results have so far been obtained, particularly with regard to organoid preparation [8, 9].

For bioethical reasons, gene therapy procedures are always performed with somatic cells through the transfer of the "therapeutic" gene using viral or non-viral vectors. Transfection efficacy tends to be higher with viral vectors (adeno-associated or lentiviral viruses) but the risk of adverse events is higher, particularly mutagenic insertion, anaphylactic reaction, and hepatotoxic damage. Given that no such thing as an ideal vector exists, a compromise must be struck between sustained long-term expression of the transgene, which entails viral integration of the host cell into the genome, and a reduction in the number of adverse events [10–12].

Gene therapy procedures can be carried out *in vivo* through systemic perfusion of the gene delivery vector, or through *ex-vivo* vector-mediated transfection and subsequent reimplantation of the patient's cells. The latter is an example of the administration of gene therapy followed by cell therapy.

Another more recent alternative is gene editing, which is based on the correction of the defective genes responsible for the patients' symptoms. This technique uses tools such as Talen, zinc fingers or CRISPR/Cas9 gene editing [13].

Other kinds of gene therapy are based on siRNA, whose function is to block RNA translation to protein by temporarily "silencing" a specific gene [14] (**Figure 1**).

As regards the other component of these types of procedures, i.e., the target cells, there is a wide range of possibilities, from pluripotent cells like induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), to multipotent cells, mesenchymal stem cells derived from adipose tissue, bone marrow, umbilical cord, or dental tissue, and differentiated adult cells [15–17].

iPSCs and ESCs are pluripotent cells that must be used with great caution due to their teratogenicity and genetic instability [18]. Use of ESCs is moreover associated with important bioethical issues [19].

Mesenchymal stem cells (MSCs) are currently the only stem cells that have not only shown themselves to be safe in several clinical trials but have also demonstrated their efficacy in a phase III clinical trial, which resulted in their use being approved by the EMA [20] for the treatment of Duchenne muscular dystrophy [21].

Although the mechanism of action of MSCs is not yet fully understood, they are believed to play a role in the process of tissue repair and regeneration, mainly due to their ability to migrate toward damaged or swollen tissues, their angiogenic capacity, their anti-infectious properties and, above all, their immunomodulating and anti-inflammatory effect resulting from the secretion of trophic factors. Moreover, they are responsible for the activation of stem cells that reside in the body and for attracting endogenous cells to the defect site.

Although it is true that one of the most promising options arising from murine models was the use of preconditioned mesenchymal stem cells with different bone morphogenic proteins, unfortunately the *in-vivo* studies performed with larger mammals and the *in-vitro* studies with human mesenchymal cells yielded disappointing results. This prompted the development of fresh research projects, dealing

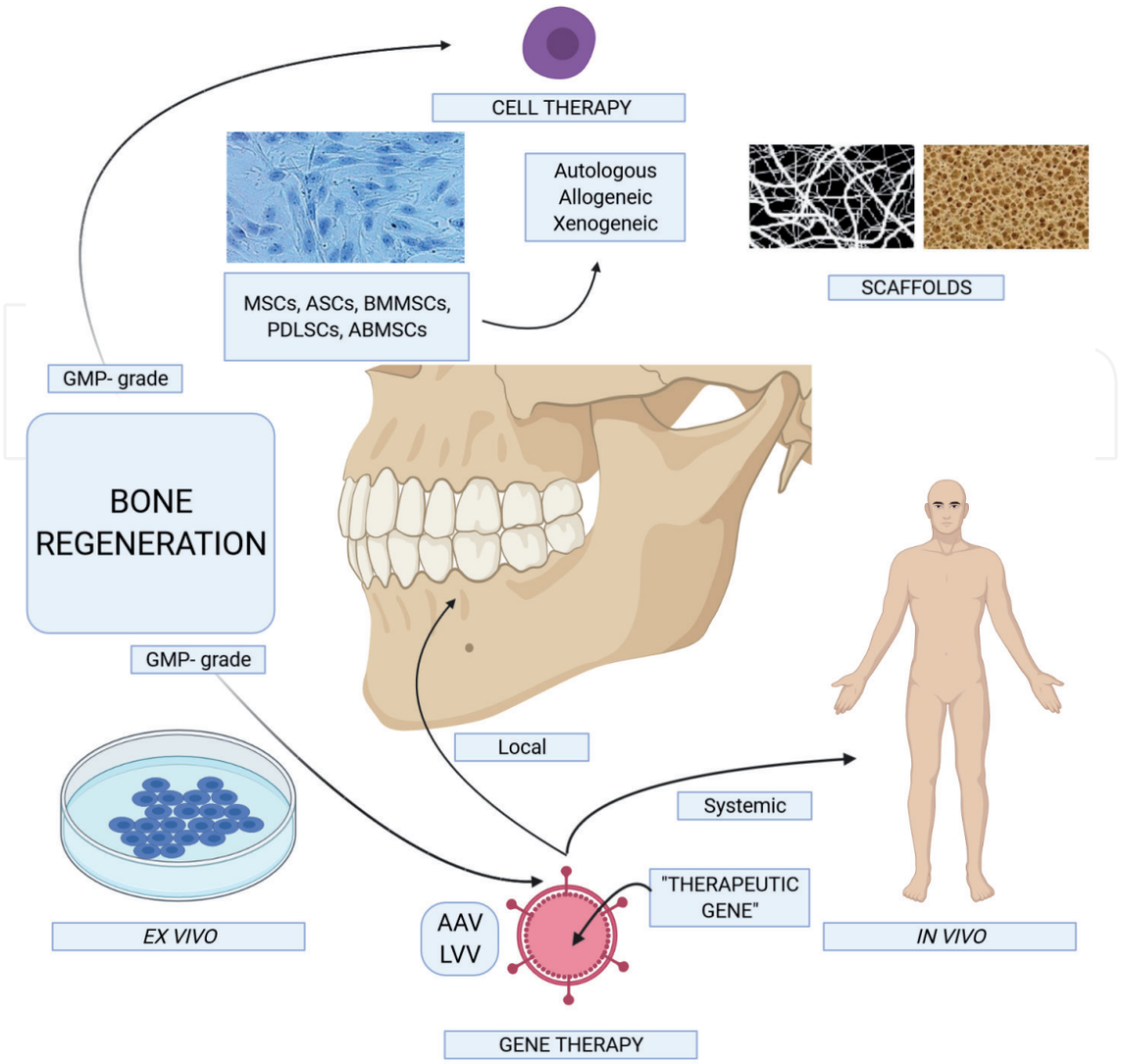

Figure 1.
Application of gene- and cell therapy to bone regeneration. GMP-grade: Good manufacturing practices-grade; MSCs: Mesenchymal stem cells; ASCs: Adipose-derived stem cells; BMMSCs: Bone marrow mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; ABMSCs: Alveolar bone-derived mesenchymal stem cells; AAV: Adeno-associated viral vectors; LVV: Lentiviral vectors. Cell therapy is one of the techniques used in bone regeneration. MSCs, ASCs, BMMSCs, PDLSCs and autologous, allogenic or xenogenic ABMSCs are used together with scaffolds. Gene therapy procedures can be carried out in vivo through systemic perfusion of the gene delivery vector (AVV, LVV), or through ex-vivo vector-mediated transfection and subsequent reimplantation of the patient's cells. (created in Biorender.com).

particularly with the use of biomaterials to control the ability of MSCs to differentiate and secrete trophic factors by generating a more favorable microenvironment. In the case of a large animal model, the induction of periodontal regeneration through the administration of locally applied growth/differentiation factors is being investigated in non-human primates. Dog models have been developed to study the application of different cells, biomaterials, and scaffolds to periodontal regeneration [22]. In addition, gene therapy protocols based on the transfection of stem cells with viral vectors have been used in mammal models to increase the expression of growth factors [23].

These cells may be isolated from different tissues such as the bone marrow, fat, the umbilical cord, dental pulp, and more recently from endometrium and menstrual blood [16, 24]. They are associated with high proliferation and self-renewal rates, they are capable of secreting countless growth factors, they are easy to obtain and characterize and, most importantly, they are capable of modulating the immune response (they have very low immunogenicity) due to the fact that they

express immunomodulating cytokines and do not express the class II major histocompatibility complex (MHC-II) or T-lymphocyte co-stimulating molecules such as CD40L, CD80 y CD86 [25].

They also display a high potential to differentiate to cells of the three germ layers, mesoderm (differentiation to adipocytes, chondrocytes, osteocytes, muscle cells and cardiac cells), endoderm (differentiation to pulmonary epithelial cells) and ectoderm (differentiation into neural cells). For all these reasons, MSCs hold a bright promise in terms of their clinical application [26, 27].

Their morphology is adherent, fibroblast-like and they express characteristic clusters of differentiation (CD) membrane markers such as CD44, CD90, CD117, CD73, CD29, CD13 and CD105. At the same time, they are negative for hematopoietic markers such as CD34, CD45, CD133 and BCRP1. They are endowed with a 46X(X/Y) karyotype that is stable both timewise and in terms of culture passages as it is able to maintain telomerase activity until passage 10. This turns MSCs into a useful cell therapy vehicle as they are exempt from genetic variability and tumorigenesis. They also express embryonic transcription factors such as Oct-4, Rex1 and GATA-4, but not embryonic stem cell markers such as SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 [28].

Despite the vast amounts of data available on mesenchymal stromal cells and on the different protocols developed for their expansion, characterization and differentiation, as well as for their manufacturing, good manufacturing practice (GMP) protocols for the process extending from the validation of MSCs to their preparation for clinical use, are still in their infancy [29].

The EMA has since 2007 considered expanded MSCs to be advanced therapy medicinal products (ATMPs) fit for clinical use [1].

Although different GMP protocols have been established concerning the isolation and expansion of MSCs, there is still little understanding of the soundness of the required preclinical protocols and their translation to research programs. In this regard, the International Society for Cellular Therapy (ISCT) has established a series of minimum criteria to define the identity and quality of these cells before they can be categorized as ATMPs [30–33]. Although there is some flexibility depending on the types of clinical trials and the regulations issued by different governments, ISCT's fundamental criteria are related with microbiological assays, endotoxin and mycoplasma testing, feasibility tests, clonogenicity, and purity and functionality analyses.

The criteria generally used to validate the use of MSCs in clinical practice are related with the following: manufacturing approval by an ethics committee and participation of authorized sites; donor selection; isolation and expansion of the cells in accordance with GMPs [clonogenicity tests (fibroblast colony-forming units); flow immunocytometry-based cell characterization; differentiation potential assays]; quality control (microbial testing, mycoplasma and endotoxin detection, karyotyping); and shipment from the manufacturing site to the clinical site where they are due to be used in the conditions required for this ATMP (temperature between 3 and 5°C and delivery time under 24 hours) [29].

2. Gene therapy in periodontal disease

As explained above, and as will be specified below, cell therapy is at present the most potentially useful tool to treat periodontal disease. However, gene therapy protocols can also make important contributions in specific cases.

Gene therapy may allow an increase in the bioavailability of certain growth factors or even some proteins that contribute to promoting the modulation of

periodontal tissue, specifically alveolar bone. The cells in the periodontal ligament (PDL) are thus characterized by several protein markers such as type III collagen, osteopontin, bone morphogenetic proteins (BMPs), osteocalcin and bone sialoprotein. Among them, BMP-4 is particularly important for bone growth and bone remodeling as it stimulates the expression of osteopontin, BMP-2 and the mRNA of osteoblast-specific transcription factor CBFA1 in human PDL cells. In this respect, Tsuchiya et al. [34] used a (highly safe) non-viral electroporation-based plasmid delivery technique to overexpress BMP-4. *In vitro* transfected rat PDL cells exhibited production and secretion of the mature form of BMP-4 without any cases of inflammation, degeneration, or necrosis.

Also, *ex-vivo* gene therapy experiments have studied the transfer of the BMP-2 gene using bone marrow-derived mesenchymal stromal cells (BMMSCs), muscle-derived cells, adipose-derived stem cells (ASCs), periodontal ligament stem cells, and fibroblasts, also showing an increase in osteogenic differentiation and mineralization [35].

Another interesting approach that used the same protocols consisted in the *in vivo* transfer of the gene that consistently expresses platelet-derived growth factor PDGF-B locally in the alveolar bone, which has been shown to stimulate the regeneration of the periodontal tissue in bone defects in rat models [36]. Periodontal lesions have been treated with a matrix containing adenovirus as a transfer vector expressing PDGF-B. Results showed higher levels of proliferating cell nuclear antigen, with positive cell staining and strong evidence of bone and cementum regeneration. A quantitative analysis showed a nearly four-fold increase in the volume of the alveolar bone and a six-fold increase in the rate of cementum repair in the areas treated with the vector.

Studies on the use of gene therapy to overexpress some proteins related to bone resorption such as osteoprotegerin [37] have shown that these proteins could constitute potential alternatives for modulating and regulating bone mass in cases of bone weakness; alveolar, mandibular, or maxillary bone osteoporosis; or where the alveolar bone needs to be bolstered prior to tooth implantation. Osteoprotegerin is a protein secreted by osteoblasts and stromal osteogenic stem cells that bears close resemblance with other members of the tumor necrosis factor family. It acts as a decoy receptor for receptor activator nuclear factor kappa-B (RANKL) and indirectly inhibits osteoclast differentiation and activation, reducing bone resorption.

In a rat model of periodontitis-derived alveolar bone resorption, non-viral gene therapy-based transfection using a subperiosteally injected osteoprotegerin gene-expressing plasmid achieved a significant reduction in alveolar bone resorption and an increase in the number of active osteoclasts [37].

More recently, gene therapy protocols are successfully being used to study other disorders that would not at first sight seem to be amenable to these techniques, such as hypophosphatasia.

Hypophosphatasia (HPP) is an uncommon hereditary disorder that affects mainly the mineralization of bones and teeth. HPP is caused by loss of function mutations (up to 388 have been reported) in the ALPL gene (chromosome 1) that expresses tissue-nonspecific alkaline phosphatase (TNALP) [38].

Insufficient levels of TNALP, an enzyme found mainly in bone, liver, and renal cells, result in elevated extracellular concentrations of inorganic pyrophosphate (PPi), pyridoxal 5′-phosphate (PLP), and phosphoethanolamine (PEA). The ensuing increase in the extracellular PPi/inorganic phosphate (Pi) relation acts as an inhibitor of bone mineralization, affecting mainly the hard dental tissue (alveolar bone) and resulting in premature tooth loss [39–41].

In general, justification for research into and subsequent application of new advanced therapies depends on the availability (or lack thereof) of an appropriate,

convenient, and safe treatment for a given condition. In the case of HPP, treatment before 2015 consisted in an attempt to mitigate symptoms by controlling calcium and phosphorus levels. Enzyme replacement therapy with asfotase alfa has gained popularity in recent years [42], although the treatment is not always effective.

As regards the new advanced therapies, the first few clinical applications corresponded to cell therapy protocols. In this respect, Cahill et al. [43] were able to correct a severe HPP phenotype by transplanting osteoblasts with high levels of TNALP. Migration of these osteoprogenitors to the affected areas of the bone was successful in converting the disease phenotype from severe to mild.

Cell therapy protocols often produce low levels of the deficient protein and, moreover, such production is systemic. For this reason, it is in many cases inevitable to resort to gene therapy protocols which, apart from allowing higher therapeutic efficacy, exhibit longer-term sustained expression levels. Furthermore, they can be applied locally, e.g., in the periodontal tissue. In this regard, Okawa et al., [44] in an *ex-vivo* gene therapy experiment whereby lentiviral vectors and BMMSCs were used with TNALP-deficient knockout mice, were able to induce alveolar bone and cementum formation in those mice, significantly in the first case and moderately in the second, thus contributing to inhibiting premature tooth exfoliation.

More recent gene therapy studies using adeno-associated vectors have shown greater promise. Following administration of the TNALP-expressing adeno-associated vector scAAV8-TNALP to TNALP-deficient knockout mice, Ikeue et al. [45], were successful in achieving enhanced growth of the mandible, the alveolar bone, and the molar roots, inducing dentoalveolar mineralization and reducing the exfoliation risk. The same authors have optimized the efficacy of the technique thereby facilitating its translation to clinical practice [46].

3. Cell therapy and the regeneration of alveolar bone

The periodontium is a complex organ made up of four mesenchymal components (gingiva, cementum, alveolar bone and the PDL), which constitute a functional unit in charge mainly of anchoring the tooth to the jawbone firmly enough to withstand the masticatory forces, and of regulating homeostasis within the oral cavity [47, 48].

The PDL plays a fundamental structural role as it connects the cementum to the alveolar bone. It is a highly vascularized cellular tissue (fibroblasts and endothelial, epithelial, neural, and undifferentiated mesenchymal cells), made up of thick collagen fibers that are inserted into the external layers of the cementum and of the alveolar bone. Dental tissue-derived mesenchymal stem cells are responsible for maintaining hemostasis across all periodontal tissues as they are capable of differentiating to cementoblasts, which give rise to the deposition of cementum; to osteoblasts, which give rise to the deposition of bone; and to fibroblasts, which foster the formation of new connective tissue [47] (**Figure 2**).

Maintenance and regeneration of alveolar bone and of tooth and implant-supporting structures are based on a balance between bone resorption and bone formation [49], which is controlled by different types of cells, signaling mechanisms, and matrix interactions. Advanced therapies, particularly cell therapy and regenerative medicine, could be considered potential tools capable of restoring that balance in soft and hard tissues, making it possible to treat traumatic, metabolic, or congenital disorders that affect periodontal tissue regeneration [50].

This chapter will succinctly cover the possibilities offered by cell therapy for the treatment of disorders related to the loss and defective regeneration of bone mass such as osteoporosis or osteonecrosis (the former understood as a systemic

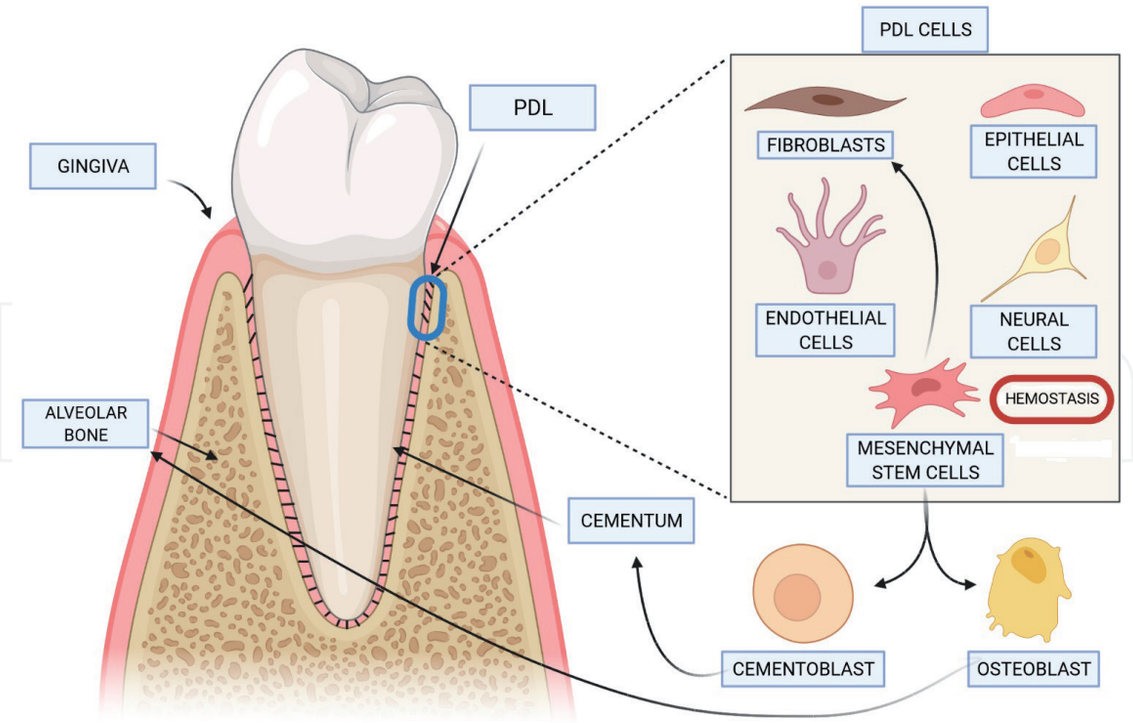

Figure 2.
Periodontium and PDL cells. The periodontium is made up of four mesenchymal components (gingiva, cementum, alveolar bone and the PDL). The PDL, which connects cementum to alveolar bone, is formed by fibroblast, epithelial cells, endothelial cells, neural cells and mesenchymal stem cells. Mesenchymal stem cells are responsible for maintaining bone hemostasis by differentiation to cementoblast, osteoblast and fibroblast. (created in Biorender.com).

metabolic disease of bone and the latter as an apoptotic loss of bone mass, particularly from the alveolar sockets), and for regenerating bone prior to placing a dental implant (osseointegration) [51].

The first pioneering studies on the regeneration of alveolar bone using cell therapy were conducted by Abukawa et al. in 2003 [52]. These authors used porcine MSCs isolated from the bone marrow, which were differentiated to osteoblasts and then incorporated to and cultured on a porous scaffold made from biodegradable poly DL-lactic co-glycolic acid. The result was the formation of bone on the scaffold's surface.

In 2004, the same authors [53] confirmed their results *in vivo* in a porcine model by using autologous constructs (cell-seeded scaffolds) to reconstruct the segmented mandible of the induced model. This resulted in the regeneration of the damaged areas of the mandible, which, in clinical, radiographic, and histologic studies, were found to contain osteoblasts, osteocytes, bone trabeculae and blood vessels.

Some time later, Streckbein et al. [54] marked a turning point in the application of cell therapy to periodontal disease. Indeed, human ASCs became a powerful cell therapy tool. ASCs were found to resemble BMMSCs in their capability of differentiating to osteocytes. Engraftment of autologous ACSs in a fibrin scaffold in a rat model resulted in the formation of significantly greater amounts of bone than in the control group.

3.1 Osteoporosis

Osteoporosis is a chronic (long-term) skeletal condition typically caused by an alteration in bone homeostasis arising from an imbalance between bone resorption and bone formation. Osteoporosis is responsible for the majority of fractures in the elderly and in post-menopausal women, who tend to experience a reduction in

bone mass and bone density. Although a genetic predisposition is undeniable, other causative factors such as the slower development of bone mass during youth as well as ethnicity, sex, lifestyle and iatrogenesis usually play a role [55, 56].

Osteoporosis is one of the main causes of alveolar, mandibular, and maxillary bone fractures as these bones are constantly subject to movements and strong masticatory forces, with the mandibular bone withstanding the greatest masticatory forces and exhibiting the most trabecular structure. It is the dentist's job to diagnose potential cases of osteoporosis as early as possible, particularly with a view to evaluating the need of implants. Current treatment for osteoporosis disorder is based on antiresorptive and anabolic drugs (oestrogens, bisphosphonates and monoclonal antibodies such as RANKL inhibitors [55].

What contribution can cell therapy make to the future treatment of this disorder? Simply put, cell therapy can offer "curative" rather than palliative treatment, restoring the structure and function of bone tissue. MSCs are the best candidates on account of their anti-inflammatory and immunologic characteristics, and of their widespread bioethical acceptance [57].

It has been suggested [58] that one of the factors leading to osteoporosis in aged bone tissue is the reduction in the number of MSCs in the bone and the resulting lower osteoblastic differentiation potential. According to several studies, this is where cell therapy would play its most evident clinical role [59–61].

The kind of osteoporosis brought about by post-menopausal estrogen deficiency exhibits very high mortality rates with an associated risk of fracture and of tooth and alveolar bone loss from the jaw. In a study aimed at evaluating the previously stated premise [58] that aging of BMMSCs contributes to the development of osteoporosis, Xu et al. [62] analyzed the effect of special AT-rich sequence-binding protein 2 (SATB2), a regulator of stemness and senescence of craniofacial bone-marrow derived mesenchymal stem cells, on ovariectomy-induced alveolar osteoporosis in a rat model. Transplantation of BMMSCs transfected with the SATB2-expressing gene ameliorated the disease phenotype, reducing cell senescence, increasing stemness and osteogenic capacity, and diminishing the number of osteoclastic markers present as well as the adipogenic potential of the cells and the *in vivo* ovariectomy-induced loss of alveolar bone.

3.2 Osteonecrosis

Osteonecrosis is a clinical entity characterized by apoptosis of the cells that make up the bone and the bone marrow. It is usually associated with the appearance of necrotic areas in the trabecular bone, the subchondral bone, and the bone marrow and, although it could affect any bone, it is most frequently found in the jawbone or, more specifically, the maxilla. Jaw osteonecrosis is an infrequent yet serious condition that involves the maxillary bone [63].

Osteonecrosis generally sets in as a result of exposure of the jawbone to the oral cavity for a period of at least 8 weeks, following which cells (usually osteocytes) become senescent and apoptotic from lack of blood supply from the gingiva. Although there is still no consensus regarding the osteopathogenesis of osteonecrosis, certain situations have been identified as potential causative mechanisms: invasive dental procedures such as tooth extraction surgery; trauma in the area of the maxillary; abnormal (spontaneous) growth of the bone in the palatal area or the internal areas of the mouth, even in patients without identifiable risk factors; radiation therapy (radiation-induced osteonecrosis); head and neck cancer; herpes zoster virus infection; steroid treatments; osteomyelitis; and chronic bone infection. Jaw osteonecrosis may remain asymptomatic for long periods of time, typical symptoms including pain in the affected area, inflammation episodes, redness,

and other signs of infection in the gingiva. Patients may experience numbness or a feeling of heaviness in the jaw, develop a purulent secretion in the area of exposed bone, exhibit intra- or extraoral fistulas, or suffer the loosening and loss of the teeth close to the affected area as a result of the weakening of the bone that anchors the teeth [64, 65].

As far as diagnosis is concerned, there is at present no predictive diagnostic test capable of determining if a patient is at risk of or predisposed to suffering jaw osteonecrosis. A number of salivary biomarkers have recently been described, which may potentially help in diagnosing and monitoring the most common oral conditions, including oral leukoplakia, oral lichen planus, Sjögren's syndrome, periodontitis, peri-implantitis, and medication-related osteonecrosis of the jaw [66]. Salivary biomarkers such as interleukins or growth factors have shown themselves useful in diagnosing and following up these conditions, making it possible to conduct an early evaluation of the risk of malignization and monitor the efficacy of treatment.

Treatments based on antiresorptive drugs such as bisphosphonates administered to patients at high risk of osteoporosis or as treatment for bone cancer have often resulted in an increase in jaw osteonecrosis [67, 68]. The mechanism by which bisphosphonates may result in maxillary osteonecrosis are currently not understood, but they have been shown to affect dentoalveolar structures, limiting or impeding bone regeneration due to inhibition of osteoclast formation and/or suppression of cell turnover.

Prevention through health education, dental hygiene and periodic dental visits from early childhood is essential. In most cases, a detailed anamnesis is crucial for both early detection and prevention [69–71]. Routine treatment is based on antibiotics, antibacterial mouthwashes such chlorhexidine, and removable oral appliances (retainers) or dental debridement.

There being currently no standard treatment to promote regeneration of the necrotized area, advanced therapies and, specifically cell therapy, are emerging as valuable tools not only to curb the necrosis but also to restore and regenerate the necrotized areas. Although studies have so far focused on bisphosphonate-induced jaw osteonecrosis, the solutions they propose – if effective – could be applied to most kinds of jaw osteonecrosis, regardless of their etiopathogenesis.

The first clinical trial that used cell therapy to attempt regeneration of the alveolar maxillary bone was conducted in 2009 by d'Aquino et al. [72]. The authors used a biocomplex consisting of autologous dental pulp stem cells and a collagen scaffold. Histological observation unambiguously showed complete regeneration of the bone at the necrotic site with optimal rehabilitation of the alveolar bone and full restoration of the periodontal tissue.

Very good alveolar bone regeneration results have also been obtained in bisphosphonate-induced osteonecrosis models using allogeneic bone marrow [73] or ASCs [74]. The advantage of ASCs, as compared to BMMSCs, lies in their easy and less invasive harvest, their higher yield, their higher proliferation and duplication potential, their lower levels of senescence; and their higher angiogenic anti-inflammatory capacity. These cells also exhibit higher survival rates in ischemic environments as well as an increased secretion of growth factors such as the vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF). These factors are required in hypoxic environments, which is useful for the treatment of osteonecrosis and bisphosphonate-related ischemic wounds derived from jaw osteonecrosis [74, 75].

There is currently only one clinical trial underway aimed at evaluating the use of cell therapy for the treatment of jaw osteonecrosis [76]. It specifically seeks to determine the safety of using autologous BMMSCs in the presence of a porous

tricalcium phosphate scaffold and in a demineralized bone matrix in patients with jaw osteonecrosis. It is a phase I, prospective non-randomized single-centre unblinded open clinical trial including patients between 18 and 85 years of age. The study is the result of a collaborative effort between the Virgen de la Arrixaca Institute of BioHealth Research (Region of Murcia), the Virgen de la Arrixaca University Hospital (Region of Murcia), the Regional Ministry of Health of Murcia and the Spanish Ministry of Health.

Patients with a definitive clinical and radiological diagnosis of jaw osteonecrosis, of whatever etiology, are implanted with the cell "construct" (MSCs, tricalcium phosphate and demineralized bone matrix). One month prior to implantation, the MSCs are first harvested from the patients' bone marrow and then characterized and expanded in GMP-grade conditions. The cells are seeded in tricalcium phosphate and maintained in culture for 14 days. On the day of implantation, the MSCs seeded in tricalcium phosphate are admixed with the demineralized bone matrix, the combination being subsequently coagulated with autologous platelet-rich plasma before engraftment can occur. Finally, the oral mucosa or the skin are closed tightly with silk sutures. A careful evaluation must be made of different circumstances related with the procedure such as bone ischemia or new bone formation; non-severe adverse events related with the procedure such as local surgical wound infections, non-union and anaphylactic reactions; time to wound healing; appearance of local pain as determined by a visual analogue scale; bone formation as measured by computerized tomography; and quality of life as measured by EuroQol-5D [77, 78].

Mesenchymal stem cells could also play an important osteogenesis and bone regeneration role in cases of osteoradionecrosis [79], a severe and difficult-to-manage complication of the jawbone following high-dose radiation therapy in cases of head and neck cancer.

3.3 Bone regeneration and osseointegration prior to dental implant placement

Placement of intraosseous implants is a routine dental procedure aimed at restoring missing teeth and masticatory function. However, the stability of implants is often compromised by the presence of an insufficient amount of supporting bone mass. Sinus elevation treatment using autologous bone and allografts is the standard alternative in these cases. The problem lies in amount of autologous bone required for such procedures, which varies as a function of the magnitude of the damage present. Moreover, alternative alloplastic materials are often ill suited to compromised vascular environments.

Initial studies in this field, such as those by Matsuo et al. [80] have made interesting contributions. Using particulate cellular bone and marrow (PCBM) and platelet-rich plasma (PRP), these authors obtained statistically significant increases in the volume of trabecular bone. Equally interesting findings were reported by Trautvetter et al. [81] who evaluated the effect of applying autologous periosteal bone grafts in conjunction with scaffolds to atrophic maxillary bone on long-term clinical restoration in one-stage procedures, which also involved placement of dental implants. At six months post-op, these authors observed the presence of trabecular bone with active osteocytes and osteoblasts and no signs of bone resorption, connective tissue formation or necrosis.

Different alternatives have been tested, with varying degrees of success in an attempt to achieve bone regeneration, including multiple biomaterials and scaffolds (both natural and artificial), different types of stem cells obtained from the dental follicle, the periodontal ligament, the dental pulp, the salivary glands and the adipose tissue; and the multiple growth factors used for dentistry applications

in the framework of tissue engineering programs. Nonetheless, developing new methodologies and strategies is required to address the problems inherent in the reconstruction of periodontal bones and tissues [82].

Further in-depth research will be required to determine the influence of the microenvironment inside the damaged periodontium on the efficacy of the new strategies that are being developed [83]. The microenvironment exerts a very significant influence on physiological and physiopathological function, and on the therapeutic effect of MSCs. The niche where these cells reside is made up of multiple cell populations, tissue components, and soluble factors that regulate the cells' behavior. The fact that viability and differentiation of MSCs are compromised in conditions such as osteoporosis and periodontitis may aggravate the patient's condition and disrupt the tissue healing process.

Studies are currently underway to investigate ways of improving and optimizing the microenvironment where transplanted cells are going to reside. These studies have used either pharmacological or epigenetic techniques [84], or cell-free (extracellular vesicle-based) applications [85] to enhance the resistance of exogenous MSCs.

In any event, whether by classical methods or by cell therapy protocols, all efforts should be aimed at achieving osseointegration to enhance implant efficiency and durability [86]. The key milestones on the road to osseointegration are as follows: firstly, it is essential to make sure that periodontal tissue responds positively to the implant from the outset; subsequently, it must induce osteogenesis and bone remodeling around the implant. As osseointegration is a process mediated by the innate immune system that involves the complement system and reactive macrophages, factors such as the design and the chemical composition of the implant, the surgical technique employed, the use of "therapeutic cells", the local microenvironment and the patient's systemic characteristics, may play a significant role.

Recourse to StemBios Cell therapy has facilitated induction of early osseointegration in primary dental implants. StemBios Cells® are pluripotent stem cells derived from adult blood and bone marrow. They are equipped with specific biomarkers and can be easily cultured *in vitro* in large quantities. They possess the advantages of embryonic stem cells but, unlike them, they are not teratogenic, and they cannot result in immunologic rejection. They are capable of differentiating to endodermal, mesodermal and ectodermal cells, both *in vivo* and *in vitro*. In an analysis of 11 subjects who had received a dental implant in the mandible only one of whom was treated with StemBios cell therapy at the time of implantation, Weng et al. [87] found that this subject exhibited superior healing of the bone tissue, particularly regarding early bone ingrowth, as compared with that observed following implantation without this kind of cell therapy.

More recently [88] it has been shown that BMMSCs play an important role in the efficacy and induction of osseointegration following dental implant placement. Uncontrolled diabetes mellitus is known to result in very poor osseointegration and reduced implant durability. Alqahtani et al. analyzed the effect of BMMSCs in the presence of platelet-rich plasma on the osseointegration of implants placed in New Zealand rabbits with induced type 1 diabetes. Implants were placed with the help of collagen sponges loaded with osteoinductive BMMSCs and platelet-rich plasma. Osseointegration was significantly more effective in the presence of BMMSCs than in implantations where only platelet-rich plasma was used.

As other tissues in the human body, periodontal tissues are endowed with a reservoir of MSCs that share the same characteristics as other mesenchymal cells such as adherence, the potential to differentiate to at least three cell lines, and specific cluster of differentiation markers for stromal cells [89]. In addition, these cells possess immunomodulating functions. Their properties make these cells potentially

applicable in clinical practice for the treatment of periodontal and other conditions, even neurodegenerative ones, such as Parkinson's disease [90].

The first mesenchymal cells isolated from periodontal tissues were human dental pulp stem cells (hDPSCs), followed by apical papilla stem cells (SCAPs), periodontal ligament stem cells (PDLSCs), gingiva-derived mesenchymal stem cells (GMSCs), dental follicle stem cells (DFSCs), tooth germ stem cells (TGSCs), and alveolar bone-derived mesenchymal stem cells (ABMSCs). PDLSCs, GMSCs, TGSCs, SCAPs and ABMSCs, the latter sharing many characteristics with BMMSCs, present osteogenic capacity. For their part, DFSCs play a role in the formation of alveolar bone and the root-bone interface in tooth development [89].

Unfortunately, the number of stromal cells that can be obtained from periodontal tissue is extremely low, which represents a significant hurdle for the use of those cells when harvested autologously (directly from the patient). This makes it necessary to use other allogeneic sources of adult tissue such as BMMSCs and, especially, ASCs, given their greater ease of harvest and greater yield [91].

As a general concept cell therapy, as used to regenerate periodontal bone tissue, is based on a combination of a cellular element, made up of the patient's own autologous mesenchymal stem cells, an extracellular element, or scaffold, that provides a substrate for tissue growth and, lastly, a series of chemical-molecular elements, mainly trophic and growth factors secreted by the cells themselves, that play a role in the regenerative process. In other words, cell therapy involves an osteogenic cellular component, a series of osteoconductive signals (trophic factors) and an osteoconductive support component (scaffold). In this regard, given that there are multiple factors that may have an impact on the success of these new therapies, the results of translating the findings of preclinical trials using *in vivo* animal models to the human clinical setting, are not always the ones initially expected. The reasons for this discrepancy are basically related with size-related differences between human and animal defects and with what is known as "diffusion distances," which have to do with limitations to massive transport (e.g., oxygen diffusion and elimination of metabolic waste), which are essential for the survival of the transplanted cells. Moreover, as mentioned above, there may exist significant differences between animal and human models in terms of the local microenvironment where the cells reside, and in terms of the epigenetic processes at work in each of them. These aspects are likely to exert a huge impact on the results obtained [83, 84]. For these reasons, hystomorphometric analysis of biopsy samples is the most effective way of quantitatively evaluating regeneration of the bone structure [92].

It should be noted that, when applying advanced therapies (MSC-based therapy), apart from measuring the efficacy of the procedure, it is essential to consider its cost-effectiveness. Although the cost involved in the expansion and preparation of cells is high in GMP-grade procedures [29], several studies [93] have confirmed that, taking into account the indirect costs related to hospitalization and complementary treatments such as general anesthesia and the higher complications rate and higher morbidity associated with traditional grafting procedures, the cost-efficiency of the new advanced therapies could be higher.

3.4 Clinical trials in progress

As mentioned above in this chapter, although we have a robust understanding of the properties of stem cells and of the viral vectors used for gene transfer, there still remains work to be done before they can be applied to clinical practice, including GMP protocols [29]. The gap will be bridged gradually as the results of the different clinical trials underway on the new or advanced therapies become available. The ISCT has already established a series of minimal safety requirements that must be

met when defining the identity and quality of these cells as ATMPs. These requirements are mainly related with the risk of contamination by pathogens, the presence of endotoxins and mycoplasma, the viability of the cells, the safety of the viral vectors and the teratogenic safety of the cells. These requirements must unfailingly be met by any clinical study that is undertaken.

One of the first successful clinical trials that used BMMSCs was performed by Gjerde et al. (ClinicalTrials.gov *identifier: NCT02751125*) [94, 95], which started enrolling patients in April 2016 and was concluded in March 2020. The trial was sponsored by the University of Bergen with the collaboration of Ulm University, Haukeland University Hospital, University of Nantes, Madrid's Complutense University, the University of Aarhus, the International University of Catalonia, Assistance Publique - Hôpitaux de Paris and the European Commission. It was a pilot project aimed at reconstructing atrophied posterior mandibular alveolar ridges by using biomaterials and autologous BMMSCs. The last step in the process was the insertion of an implant into the newly formed bone. This was an interventional clinical trial with 13 subjects, between 18 and 80 years (**Figure 3**).

Cells were obtained from the bone marrow of the patients' alveolar ridge. The sample was processed in a cell therapy lab using GMP-grade protocols. Cells were expanded and characterized through flow immunocytometry and, 21 days later, they were transplanted to the subjects' alveolar bone. Before closing the transplant site, the cells were brought into contact with dicalcium phosphate (DCP) and the material was covered with a reinforced titanium membrane. From four to six months later, the bone was biopsied and implants placed in the regenerated bone. Patients were followed up for 1, 2, 3 and 5 years to assess the stability of the implant. Moreover, the newly formed bone was clinically and radiologically assessed. Implant stability was measured using the Ostell™ system, based on Resonance Frequency Analysis (RFA) [96]. In addition, an evaluation was made of potential adverse events derived from the treatment in general and of the cells in particular (safety and tolerability).

Results showed that clinical reconstruction of the alveolar ridge is a feasible and safe procedure yielding a predictable outcome. Osseointegration was achieved in all dental implants.

A promising clinical trial (ClinicalTrials.gov *identifier: NCT04297813*) got underway in March 2020. It is meant to be the first controlled trial using autologous mesenchymal stem cells, cultured, expanded, and maintained using synthetic biomaterials with the aim of regenerating enough maxillary bone to offer support to dental implants. The coordinators of the study are Pierre Layrolle, from the University of Nantes (France) and Kamal Mustafa, from the University of Bergen (Norway) and it is sponsored by the European Union (H2020 Maxibone Project). This clinical trial follows on from a previous study by Gjerde et al. [94, 95].

It is a phase III interventional multicentre randomized controlled clinical trial of 150 patients over 18 years of age. It is aimed at comparing the safety and efficacy of using autologous mesenchymal stem cells cultured on calcium phosphate biomaterials against the use of autologous bone grafts. Patients have been randomized into either a control group, where subjects receive standard treatment (a jawbone graft), or an experimental group, where subjects receive a combination of biomaterials and cultured and expanded autologous stem cells [97].

The cells are obtained from the patients' coxal bone marrow. These stem cells are being expanded and produced in two different labs, one at the Transfusional and Immunogenetic Medicine Institute of the University of Ulm (Germany), and the other at the Créteil Centre de Thérapie Cellulaire (France). After two weeks, the mesenchymal stem cells are sent to a surgical clinical centre where they are brought into contact with a biomaterial (DCP) and, subsequently, engrafted onto

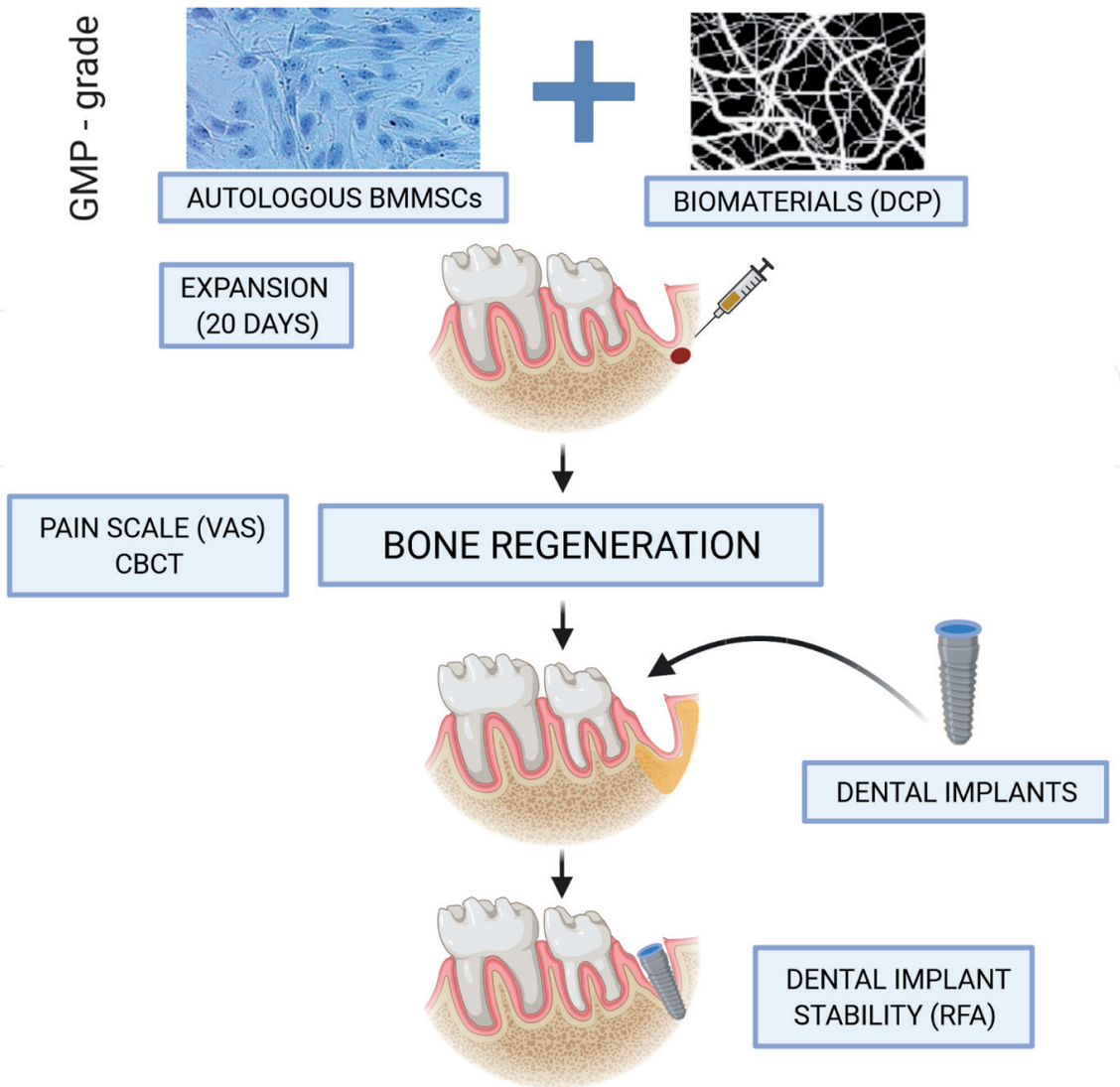

Figure 3.
Clinical trials currently in progress. GMP-grade: good manufacturing practices-grade; BMMSCs: bone marrow mesenchymal stem cells; DCP: dicalcium phosphate; VAS: visual analog scale; CBCT: cone beam computed tomography; RFA: resonance frequency analysis. These clinical trials are based on the use of BMMSCs together with dicalcium phosphate (DCP). The objective was to reconstruct atrophied posterior mandibular alveolar ridges by using biomaterials and autologous BMMSCs. The last step in the process was the insertion of an implant into the newly formed bone. Patients were followed clinically and radiologically. (Created in Biorender.com).

the maxillary and mandibular alveolar ridges. Patients in the control group are engrafted with autologous bone from the posterior mandibular ramus. A non-resorbable membrane is used to cover the grafts and guide the tissue regeneration process. Five months later, the implants are placed following an assessment of the amount of bone regeneration obtained. Bone biopsies are then obtained, which are evaluated by synchrotron, microcomputerised tomography and histology. Subsequently, once dental implants have become osteointegrated, the protective elements can be placed.

The primary outcome measure in this study will be an evaluation of the changes in the linear measurements of the alveolar bone width, as measured below the alveolar ridge immediately before placement of the implant. Secondary outcome measures will consist, on the one hand, in a VAS evaluation of postoperative pain following each of the two treatments [98] and, on the other, of radiological analyses to determine the bone volume present, thus gauging the amount of new bone obtained. This will allow making an informed decision regarding the possibility of

placing an implant in the reconstructed area. Such as decision will be made on the basis of 3D Cone Beam Computed Tomography (CBCT) [99].

To be included in the trial subjects have to be healthy, non-smokers and in need of implants in the upper or lower jaw, with loss of vertical height and less than 4 mm lateral width. Exclusion criteria include the general contraindications for dental and/or surgical treatment, and for harvesting bone marrow specimens or bone grafts; a history of any malignant disease; previous or concurrent radiation therapy of the head and neck; a history of infectious diseases (HIV, syphilis, hepatitis B or C); uncontrolled diabetes mellitus; inflammatory or autoimmune diseases of the oral cavity; and previous or concurrent immunosuppressive treatment or high-dose bisphosphonate or corticosteroid therapy.

4. Reflections on the application of advanced therapies in dentistry

Advanced therapies encompass a wide-ranging series of different therapeutic procedures that can be applied to most conditions, transmissible or otherwise [100]. They are multidisciplinary procedures that may involve basic molecular and cellular biology techniques and tissue engineering [101]. In general, implementation of a new healthcare technology, understood in this case as a new therapeutic procedure, has vast repercussions not only from the clinical point of view but also from a social and economic perspective. This places a heavy responsibility on the shoulders of managers and officials in charge of its application. To this should be added human innate reluctance to adopt new ideas especially when, as in the case of the new therapies, there are so many unknowns regarding potential medium and long-term effects. However, some clinical disciplines are more open to change than others. This should be considered together with the social, legal and bioethical considerations associated to the introduction of any new technology [102–104].

An explanation must be found to the fact that despite the significant progress made and the success obtained by research into ATMPs in the last few years, the number of procedures and products authorized by evaluation agencies has been extremely low and limited to relatively severe conditions. Going back to dentistry, although significant improvements have been made on the more classical techniques and on advanced therapies, much work remains to be done before many of these procedures become standard clinical practice. The reasons for this may be related to the novelty of the techniques themselves or to other factors of a social, economic, or bioethical-legal nature, without mentioning the inherent distrust of practitioners and the general public toward these innovative procedures. Indeed, even if dental conditions are not in principle potentially fatal and may often be prevented, they are highly prevalent, affecting more than 3,5 billion people worldwide [105]. They are therefore associated with a high economic cost and, very often, a low quality of life. They are also a social problem as they typically affect the less affluent social classes [106]. Moreover, dental infections could result in a worsening of systemic conditions, such as cardiovascular disease [107, 108]. For all these reasons, any procedure that may ensure greater medium- and long-term efficacy of dental procedures should be eagerly embraced.

What could then be the reasons behind the low number of patients enrolled in advanced therapy programs in routine periodontal practice? The first explanation, applicable to any type of condition except severe or fatal ones, is the lack of a sufficient number of preclinical and clinical trials to confirm the therapy's efficacy and, particularly, its safety. As advanced therapy procedures were introduced only recently, their potential, especially long-term, adverse events are not known. It is therefore essential for a whole body of new clinical trials to be undertaken to dispel

misgivings and promote confidence among patients. This will inevitably require greater economic investments in research.

As far as the safety of these protocols is concerned, the more stringent requirements imposed by regulatory agencies typically results in a delay in the implementation of these new therapies. In this regard, special programmes have been introduced in the last few years to accelerate regulatory procedures and overcome the so-called "valley of death," which tends to hold up the deployment of these novel procedures in clinical practice [109–113].

ATMPs are regulated in the European Union by Directives 1394/2007 [114] and 2009/120/EC [115], which amended Directive 2001/83/EC. Those products are controlled by the Committee for Advanced Therapies and the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMA), the European equivalent of the United States' Food and Drug Administration (FDA). The drug regulatory agencies of Australia [116], Canada [117], the United States [118], Korea [119], Singapore [120] and Japan [121] have developed a specific framework for regulating their use [122].

Meeting the requirements for the accreditation, designation, authorization, or licensing of the tissue establishments and cell preparation processes involved in advanced therapy medicinal products can be challenging. As explained above, the main goal is to ensure that such processes are safe. However, it must be considered that regulatory agencies prefer to tread cautiously into this uncharted territory so full of legal loopholes. Apart from addressing those loopholes, which is the responsibility of the legislative bodies, an attempt should be made at harmonizing the safety norms applicable to advanced therapy medicinal products across the different regulatory agencies, particularly concerning their general principles, which seem to be more skewed toward ensuring patient safety than facilitating the development of innovative therapies to address current medical challenges. This is of course complicated by the fact that the handling and manipulation of cells and tissue materials must be performed under Good Manufacturing Practice (GMP) conditions as regulated by Directive 2003/94/EC [123].

It is therefore necessary to find a compromise between the three terms in the equation: safety, economic investments, and cost-efficiency. In severe or fatal conditions cost-efficiency tends to be very high but in others such as dental conditions, which are typically non-fatal, there being other longer-standing and more economical alternatives cost-efficiency is usually much lower. A consensus must be reached between academia, industry, regulatory authorities, and other stakeholders that paves the way to improving the design of ATMPs, facilitating their use, and making it easier for them to make the transition from bench to bedside. In other words, regulation, reimbursement, and realization are the 3 Rs [124] required to ensure that patients can benefit from advanced therapies in a safe and efficient way.

It could be argued that the lack of correlation between novel ideas and therapeutic procedures on the one hand, and clinical practice on the other, is generally due not so much to scientific reasons but rather to regulatory and cost-related ones. It must be considered that advanced therapies are essentially personalized rather than one-size-fits-all therapies, which inevitably leads to higher design and production costs [125].

It should not be forgotten that the rate of progress in a given clinical domain is determined by the importance assigned by governments and societies to advances in that domain. Indeed, implementation of novel technologies is heavily influenced by their usefulness in the eyes of society, particularly in the face of an ever-increasing life expectancy, which inevitable leads to a rise in the number of comorbidities. From a dentistry perspective, dental problems resulting from a longer life expectancy lead to a lower quality of life because of a disturbed masticatory function.

Priorities in the realm of dentistry are also influenced by the idiosyncrasies and the mindsets of the different countries and geographical regions with respect to healthcare policy. In some Western countries, dental care and hygiene have been considered to play a secondary role, which has resulted in a lack of awareness of the importance of dental education and an ensuing lack of prevention programs. Some governments do not seem willing to devote the required resources to address conditions that might not appear excessively severe at first glance, but which result in significant direct and indirect social costs in the medium or long-term.

Implementation of new therapeutic strategies and procedures is clearly constrained by the level of priority given to specific clinical areas, starting at the base of the pyramid, which is prevention-oriented health education. In dentistry, as well as in other areas, it is essential to support research into new therapies based on ATMPs in order to provide patients with alternative dental treatments that are safer and more effective in the long term.

5. Conclusions

- The topic addressed in this chapter, i.e., the contribution of gene and cell therapy to dental tissue regeneration, is closely related to developmental dental defects and their treatment. Protocols based on gene and cell therapy represent "curative" rather than "palliative" therapeutic tools for defects affecting human dental hard tissues. Proteomics, genomics, and biomaterials science will be instrumental for translating these strategies into clinical practice.

- Advanced therapies constitute potentially essential strategies for the treatment of periodontal disease.

- Gene therapy, through adeno-associated or lentiviral vectors could activate periodontal tissue and alveolar bone modulation through the transfer of "therapeutic" genes expressing proteins such as BMPs, osteoprotegerin, or tissue- nonspecific alkaline phosphatase, which is deficient in patients with HPP (a condition that affects mineralization of teeth and bone) among others. Those genes could also express factors such as the platelet-derived growth factor.

- In cell therapy, mesenchymal stem cells implemented in an autologous, allogeneic or xenogeneic manner, with the aid of scaffolds to enable the required three-dimensional environment, have been shown by several clinical trials to have a significant bone regeneration potential in the context of osteoporosis, osteonecrosis or alveolar bone regeneration (osseointegration) prior to placement of a dental implant. Use of these cells is safe as they do not present with teratogenicity, they have immunomodulating properties, and they do not pose the risk of immune rejection. The implanted cells maintain homeostasis across all periodontal tissues and are capable of preserving and regenerating the alveolar bone and the tooth and implant supporting structures by managing the balance between bone formation and bone resorption. The results obtained have thus far offered significant promise in terms of the long-term durability of implants given the efficacy observed in the induction of osseointegration.

- Implementation of the new therapies will require finding a compromise between safety, economic investments, and cost-efficiency. It will be necessary to reach a consensus between academia, industry, and the regulatory

authorities to improve the design of ATMPs, facilitate their use and speed up their translation to clinical practice.

- Advanced therapies are by definition personalized, which increases the costs inherent in their design and their production. For that reason, it will be essential going forward to devote greater resources to the clinical areas where they have shown greatest promise. As regards the implementation of advanced therapies in dentistry, it will be necessary to raise people's awareness about the importance of good dental health because as the investments required by medical progress and these are heavily influenced by the priorities of governments and society at large. A greater awareness will contribute to promoting innovation, efficient treatments, medium- and long-term savings, and a higher quality of life.

Conflict of interest

The authors declare no conflict of interest.

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